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Original

Roles of Bamboo *O*-methyltransferase in the Lignin Biosynthesis*

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Abstract——Bamboo *O*-methyltransferase (OMT) was purified 97-fold by ammonium sulfate fractionation, and chromatography on DEAE-cellulose, Sephadex G-200 and -100 columns and analyzed by polyacrylamide gel electrophoresis and isoelectric forcusing. Two methylation processes, i.e. caffeate to ferulate (FA) and 5-hydroxyferulate to sinapate (SA), were demonstrated to be catalyzed by the same enzyme in the lignin biosynthesis.

Bamboo OMT catalyzed the methylation of caffeate, 5-hydroxyferulate, 3,4,5-trihydroxycinnamate, 5-hydroxyvanillin, protocatechuate, but no or little methylation of chlorogenate, isoferulate, *m*-, *p*-coumarate, 3,4-dihydroxyphenylacetate, 3,4-dihydroxymanderate, gallate, pyrocatechol, pyrocatechol phthalein, and *d*-catechin. Km values for caffeate and 5-hydroxyferulate were 5×10^{-5} and 10^{-5} M, respectively, and the former methylation was competitively inhibited by the latter phenolic substrate. The enzyme was an acidic protein with pI 4.61 at 4°C, and showed optimal pH at 8.0 with half maximal activities at pH 8.6 ± 0.2 and 6.4 ± 0.2. The ratio SA/FA in Gramineae and allied species was ca. 1.0 which was smaller than that of common angiosperm OMTs. This paper will discuss two possible regulatory mechanisms in the lignin biosynthesis which might be finely controlled by the OMT.

Introduction

We have investigated *O*-methyltransferases (OMTs) in relation to lignin biosynthesis, and found that the enzyme could be roughly classified into gymnosperm- and angiosperm-type[1]. The first-type OMT catalyzes the formation of guaiacyl-unit but scarcely does that of syringyl-unit. Most of the gymnosperm enzymes belong to this type. *Pinus* OMT was reported to be a typical one[3,8], while *Thuja* OMT was shown to give rather high syringyl-unit formation which was discussed in relation to *Thuja*-lignan biosynthesis[17]. The second-type OMT catalyzes both guaiacyl- and syringyl-units formation. Most angiosperm-OMTs belong to this type which gives preferential formation of the latter-unit. A few OMTs were characterized in relation to both guaiacyl- and syringyl-units formation in soybean suspension cells[4], swede roots[5], mistletoe shoots[11], aspen-differentiating xylem[19], and other species[2,10]. They belong to typical angiosperm-OMTs except for the swede root-OMT which

* This paper is a complement of the publication in reference 2).

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catalyzes relatively little syringyl-unit formation.

The formation ratio of sinapate (SA) to ferulate (FA) by the enzyme, i.e. SA/FA ratio, is relatively correlated with the ratio of syringaldehyde to vanillin (S/V), which are lignin degradation products by alkaline nitrobenzene oxidation. The SA/FA ratio more directly reflects genetic informations comparing with the S/V ratio, thus being useful for phylogenic plant classification[1].

In lignin biosynthetic pathway, ferulate-5-hydroxylase has not been isolated and the distribution of sinapate: CoA ligase in angiosperms has been reported not to be universal. The situation indicates that the biosynthetic pathway for syringyl lignin is not conclusively elucidated and is making doubt on general SA-participation in syringyl lignin biosynthesis.

However, Gramineae is considered to utilize SA as an intermediate, because of the presence of sinapate: CoA ligase[12], efficient incorporation of sinapate into lignins[18], and universal distribution of free and esterified sinapate in Gramineae. It seems, therefore, that the formation of syringyl to guaiacyl lignin is under control of the SA/FA ratio in bamboo. Bamboo OMT which belongs to angiosperm-type catalyzes the formation of both guaiacyl- and syringyl-units at nearly same rates[1,2,9,16]. This paper forcuses on the control mechanism of the both units formation by bamboo OMT.

Results

General Properties of Bamboo OMT

Bamboo OMT was extracted from *Phyllostachys pubescens* young shoots, which showed specific activity of ca. 3 pKat per mg protein for FA-activity. The properties of OMT in crude preparation is summarized as follows. The crude enzyme showed optimal pH ca. 8.0 for both FA- and SA-activities with half maximal activities at pH 8.6 ± 0.2 and 6.4 ± 0.2 (see also in ref. 21). PCMB (3 mM) and EDTA (10 mM) moderately inhibited the enzyme activity (10% and 20% inhibition, respectively), but monoiodoacetate (0.3 and 3 mM) showed no significant inhibition[9]. EDTA and SH reagents prevented its deactivation when the enzyme was precipitated by ammonium sulfate[9]. Mg^{2+} ion and SH group seem to be involved in the enzymic methylation[9]. In addition, purified OMT was found to be an acidic protein with an isoelectric point at pH 4.61 at 4°C as shown in Figures 3 and 4.

The Ratio of SA- to FA-activities in the Crude Preparation

The ratio of SA- to FA-activities were found to be somewhat varied, when the crude OMT was assayed in different hydrogen ion concentrations. The fractions obtained by ammonium sulfate precipitation also showed the ratio-fluctuation. Thus, the crude bamboo OMT concentrated by ammonium sulfate precipitation was preliminary examined by chromatography on the OMT-polymorphism. Figure 1 and

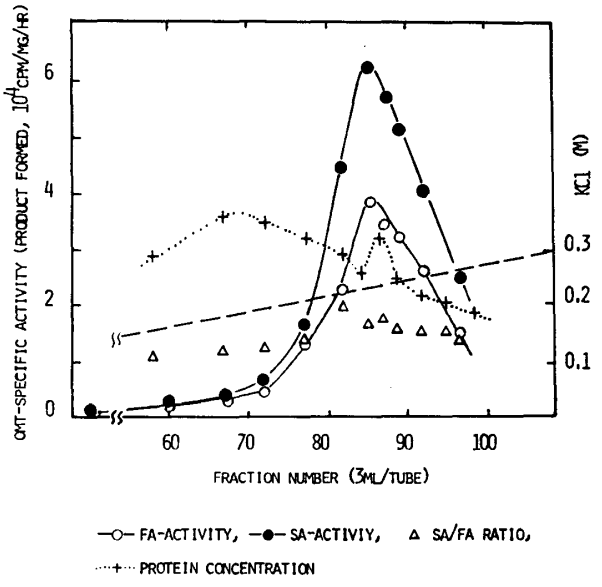

Fig. 1. Elution Pattern of Bamboo OMT on a DEAE-cellulose Column

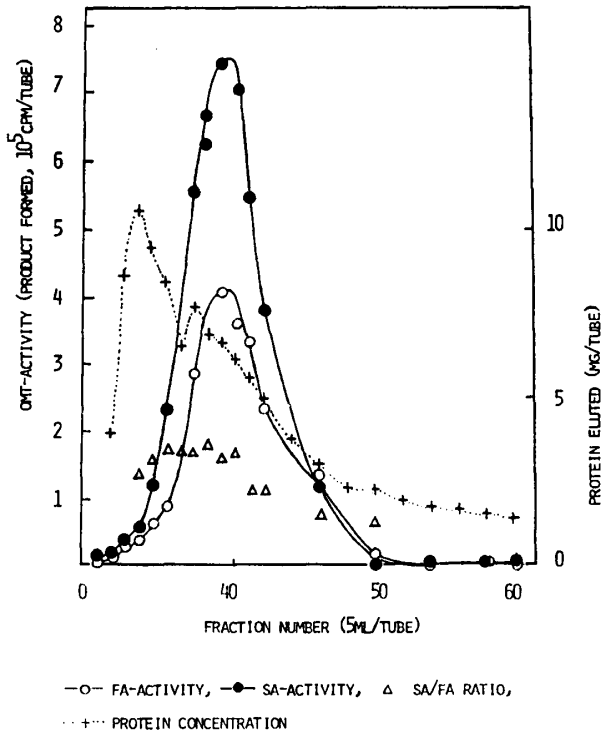

Fig. 2. Gel Filtration Pattern of Bamboo OMT on Sephadex G100

2 showed the elution profile of crude enzyme on DEAE-cellulose and Sephadex G-200 chromatography, where neither OMT-polymorphism nor dissolution of the FA- and SA-activities were observed. The apparent SA/FA ratio tends to decrease from near the OMT peak to its base in the chromatograms.

Partial Purification of Bamboo OMT

Bamboo OMT was examined by purification if FA- and SA-activities are resolved. Table 1 summarizes two series of purification achieved. Bamboo OMT was rather

Table 1. Purification of Bamboo *O*-methyltransferase

Purification of *O*-methyltransferase from bamboo (1)

Purification procedure	Total protein (mg)	Total activity (units)		Specific activity (units/mg)		Recovery(%)		SA/FA ratio	Fold
		FA	SA	FA	SA	FA	SA		
1. 0-70% Am_2SO_4	2900	5980	7300	2.06	2.52	100	100	1.22	1
2. 20-55% Am_2SO_4	1300	6890	8640	5.3	6.8	118	118	1.26	3
3. DEAE-cellulose	118	5480	6870	46.5	58.3	92	93	1.25	23
4. Sephadex G-200	65	3720	4180	57.5	64.0	63	57	1.11	28
5. DEAE-cellulose	1.5	300	368	200.0	244.0	5	5	1.22	97

Purification of *O*-methyltransferase from bamboo (2)

Purification procedure	Total protein (mg)	Total activity (units)		Specific activity (units/mg)		Recovery(%)		SA/FA ratio	Fold
		FA	SA	FA	SA	FA	SA		
1. 0-70% Am_2SO_4	1590	2600	3190	1.6	2.0	100	100	1.22	1
2. 20-55% Am_2SO_4	695	2360	2980	3.4	4.3	91	93	1.26	2
3. DEAE-cellulose	92	1210	1630	13.2	17.7	47	51	1.34	9
4. Sephadex G-100	13	396	520	30.0	39.5	15	16	1.31	20
5. DEAE-cellulose	1.4	165	195	117.0	138.0	6	6	1.18	69

stable, in contrast to the enzymes of aspen cambial zone and developing xylem[19]. Ammonium sulfate fraction at step 2, precipitated by 55%- to 20%-saturated ammonium sulfate, contained ca. 90% of the OMT activity. Elution profiles of the chromatograms during the purification were almost identical to the crude ones. The bamboo OMT was finally purified 97-fold with 5% recovery, specific activities of which were 2.50 and 3.05 n Kat per mg protein for FA- and SA-activities, respectively. The ratio SA/FA kept constant during the purification, and was found to be 1.25 ± 0.1 at pH 8.0. The final preparation was further examined by electrophoresis and isoelectric forcusing, if the FA- and SA-activities were resolved, and/or showed multiple forms. FA- and SA-activities showed a single peak at the same place as shown in Figures 3 and 4, and these accurate methods conclusively demonstrated no resolution of the both activities.

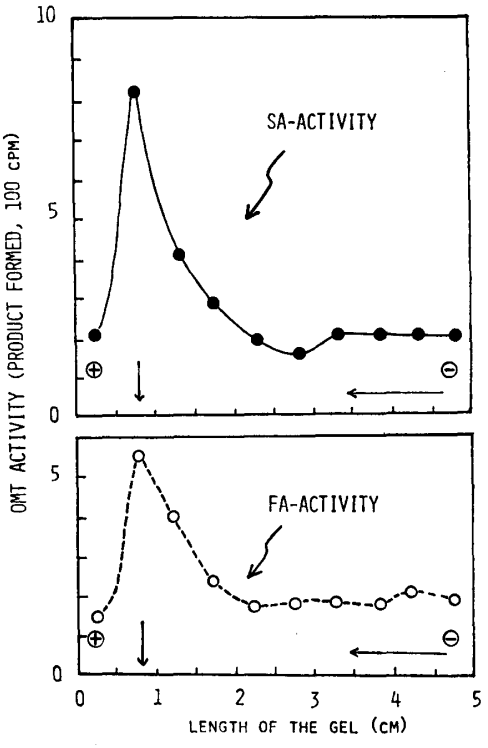

Fig. 3. Electrophoresis of Bamboo OMT on Polyacrylamide Gel

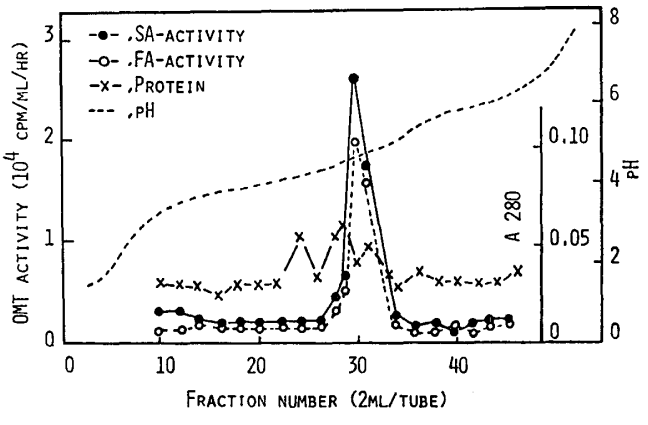

Fig. 4. Isoelectric Focusing Pattern of Bamboo OMT

Thermo-stability of the Bamboo OMT

FA- and SA-activities were similarly decreased by 10 minute-incubation at various temperatures (Figure 5C and 5D). The both deactivation-time courses were almost identical at 50°C, but with a small difference at 45°C in both presence and absence of the phenolic substrates (Figure 5A and 5B, Table 2). The heat treatment

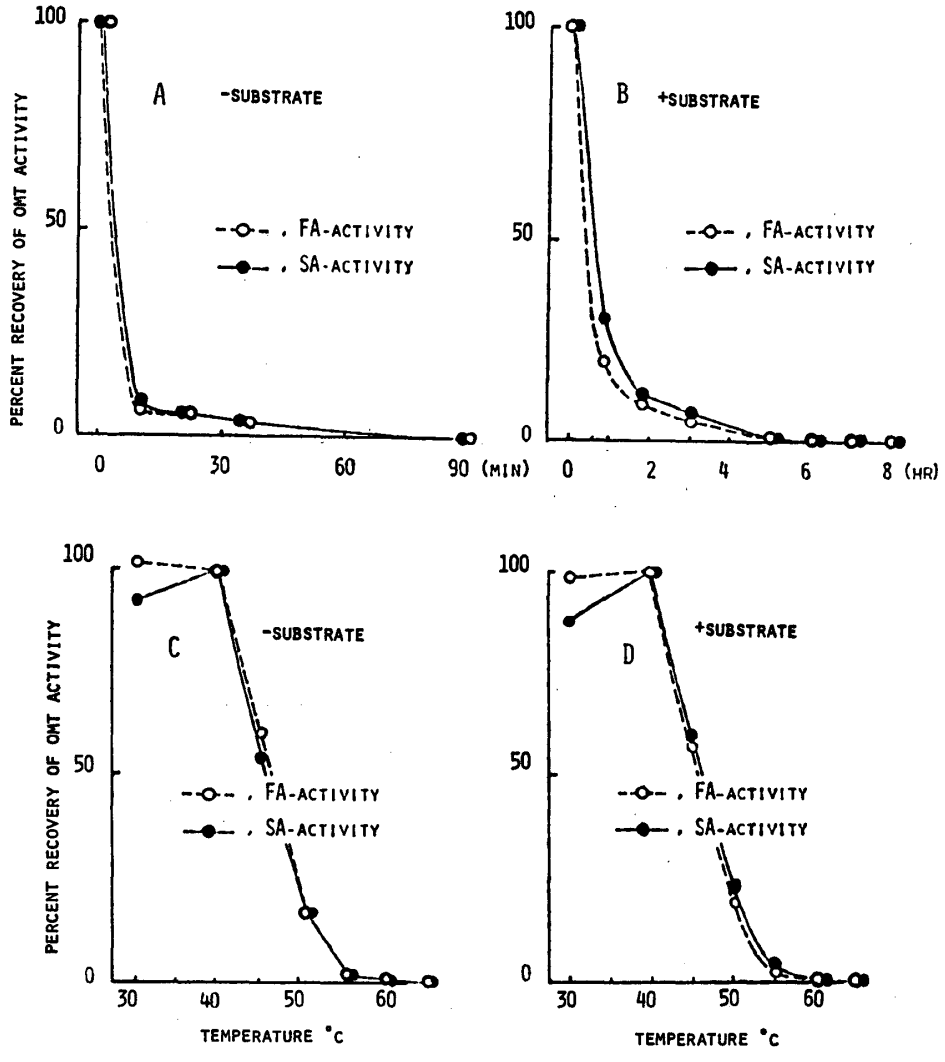

Fig. 5. Heat Treatment of Bamboo OMT
A and B: Effect of caffeate or 5-hydroxyferulate on the thermostability of bamboo OMT during heat treatment at 50°C. C and D: Denaturation patterns of bamboo OMT on 10 minutes heat treatment in the presence and absence of caffeate and 5-hydroxyferulate

Table 2. Thermostability of Bamboo OMT at 45°C in the Presence or Absence of Phenolic Substrates

Time min.	Minus substrates (cpm)			Plus substrates (cpm)		
	FA	SA	SA/FA	FA	SA	SA/FA
0	22,630	31,530	1.39	25,130	30,930	1.23
10	10,510	13,310	1.27	13,510	18,010	1.33
20	7,300	11,430	1.57	9,530	19,030	2.00
30	8,030	8,630	1.07	10,830	16,030	1.48
40	8,340	9,640	1.16	11,240	17,940	1.60
50	7,170	8,570	1.20	10,670	15,870	1.49
100	4,090	5,090	1.24	6,190	7,390	1.19

at 45°C changed SA/FA ratio which was higher in the presence of substrates than in the absence (Table 2). The half-life period at 50°C was found to be six times longer in the presence of substrates than in the absence as shown in Figure 5A and 5B. Protecting effect of 5-hydroxyferulate on the heat treatment was remarkable comparing with that of caffeate although the former substrate was not effective on the enzyme stabilization when incubated at 4°C for 5 days. The OMT-stability at 4°C was improved by adding glycerol which kept 96% of the activity during the 5-day-incubation at 4°C, while no glycerol kept 74% of the activity during the incubation.

Substrate Specificities of Bamboo OMT

Methylation by bamboo OMT was found in caffeate, 5-hydroxyferulate, 3,4,5-trihydroxycinnamate, 5-hydroxyvanillin, protocatechuate, but no or little methylation in chlorogenate, isoferulate, *m*-, *p*-coumarate, 3,4-dihydroxyphenylacetate, 3,4-dihydroxyphenylmanderate, gallate, pyrocatechol, pyrocatechol phthalein and *d*-

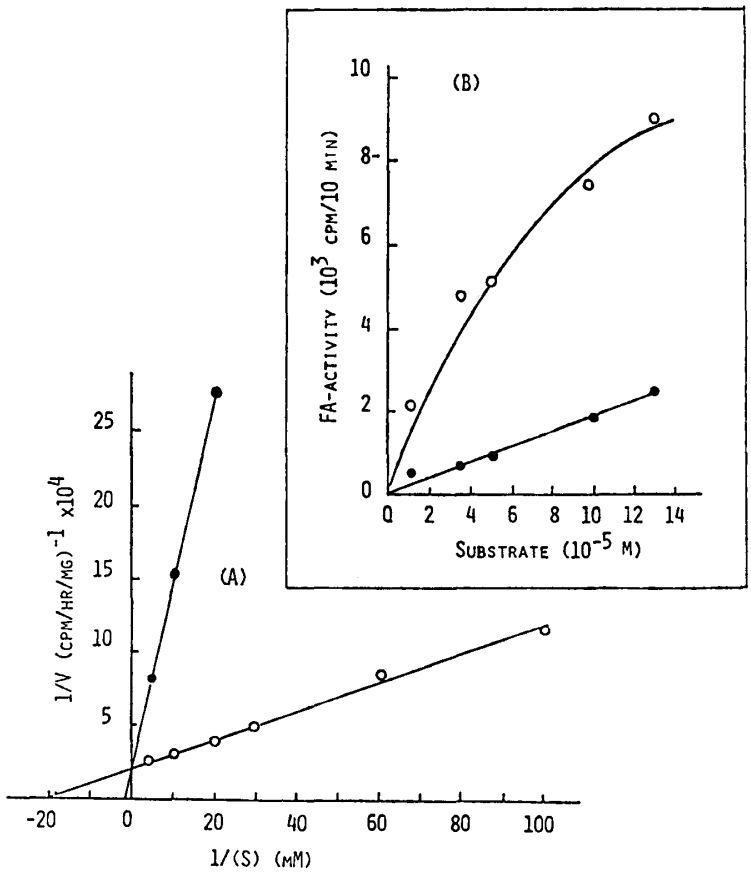

Fig. 6. Competitive Inhibition of FA-activity by 5-hydroxyferualte
(A) Reciprocal plot of methylation velocity against caffeate concentration in the presence and absence of 5-hydroxyferulate (10^{-4}M), 70% ammonium sulfate ppt, passed through Sephadex G100 (B) Effect of 5-hydroxyferulate (4×10^{-5}M) on the methylation velocity for caffeate, (32-fold purified enzyme was used)

catechin. Km values for caffeate and 5-hydroxyferulate were 5×10^{-5} and 10^{-5} M, respectively, in excess *S*-adenoxyl-L-methionine. Figure 6 shows that the latter phenolic substrate competitively inhibited FA-formation, for which Ki was identical to the Km for the same subtrate.

Discussion

Bamboo OMT is chromatographically demonstrated to be a single enzyme which catalyzes FA- and SA-formations with preferential latter formation. This is supported by remaining a constant SA/FA ratio during the purification, and by deactivation pattern on heat treatment. The finding that caffeate and 5-hydroxyferulate were competitively methylated also suggests that the bamboo OMT is a single emzyme which has one active methylating-center for FA- and SA-formation. Although apparent polymorphism had been reported on a previous work[16], no OMT-polymorphism was observed in the present experiments.

Some variation of SA/FA ratio was observed in the crude enzyme preparation possibly due to the following reasons. When the OMT-activity decreases to a control value, the SA/FA ratio gradually decreases and comes close to one, and such a low activity causes experimental error, resulting in the ratio-flactuation. Another reason is due to low protein concentration. When crude preparation contains small amounts of protein (less than ca. 80 ng), the apparent ratio was also varied (data not shown).

The facts described above partially accounts for the variation, but the flactuation of the ratio may also occur by partial denaturation of the enzyme by heat, acid or alkali, and ammonium sulfate, causing its conformational change. It is concievable that such modifications which may follow the cytoplasmic alterations occur *in vivo*, e.g. temperature, pH changes, and protease attack etc., may cause the variation of SA/FA ratio. In other words, OMT might finely change its substrate specificity via cytoplasmic alterations to adjust guaiacyl- and syringyl-formations in lignin biosynthesis.

The kinetic study demonstrated that both FA- and SA-formations were competitively controlled, suggesting that *in vivo*, a feedback inhibition might operate on the former formation by the latter substrate.

Two multiple forms were found in soybean *p*-coumarate: CoA ligase[20] and cinnamyl alcohol dehydrogenase[21], which respectively correspond to catalyze only guaiacyl group or both guaiacyl and syringyl groups. Syringyl lignin increases during xylem differntiation in angiosperm[6,13], which seems to be under control by the enzymes involved in the reduction of sinapate to sinapyl alcohol[6]. Induction of the enzymes is regarded as a coarse adjustment in the biosynthesis of syringyl lignin.

Because syringyl lignin gradually increase during the tissue differentiation, the adjustment take rather long period of time. While the bamboo OMT seems to regulate lignin biosynthesis by means of fine adjustment, i.e. the conformational change and the feedback inhibition as described above, which are capable of very minute alterations.

Gymnosperm OMT is not able to catalyze SA-formation (SA/FA ratio ca. 0.1), while angiosperm OMT catalyzes both FA- and SA-formations (SA/FA ratio ca. 3). The finding that the SA/FA ratio showed ca. one in bamboo OMT seems to be characteristic for Gramineae and allied species, e.g. *Oryza sativa* 0.9, *Triticum aestivum* 1.0, *Zisania latifolia* 1.1, *Sparganium stoloniferum* 1.5. Considering the ratio of syringaldehyde to vanillin, produced by nitrobenzene oxidation of lignin, Gramineae OMT seems to form another group.

The SA/FA ratio is found to be one of the important step in the formation of guaiacyl and syringyl lignin and could be used as a marker for phylogenic evolution in plants.

Experimental

Materials

The buffer used were followings. Buffer A: 0.2 M potassium(K)-phosphate buffer (pH 8.0), containing each 5 mM of 2-mercaptoethanol (ME), CySH, NaN_3 and isoascorbate. Buffer B: 0.5 M K-phosphate (pH 8.0). Buffer C: 0.02 M K-phosphate buffer (pH 7.4), containing each 5 mM of CySH and ME. Scintillator for radioactive counting contains toluene•2,5-diphenlyoxazole•1,4-bis-[2-(4-methyl-5-phenyloxazole] benzene (1.0$_l$: 4 g: 0.1 g). *S*-Adenosyl-L-methionine-^{14}C (SAM; specific activity 53.1 μCi/μmole) was purchased from New England Nuclear and diluted with cold one for the enzyme assay. Ampholine Carrier Ampholytes (LKB-Produkter AB; 40% w/w, pH 3–6) was obtained from American Commercial Co. Ltd., Japan.

OMT assay

Standard reaction mixture (total volume 1.0 ml) contains: Buffer B (50 μmole), CySH (10 μmole), ME (10 μmole), isoascorbate (0.5 μmole), enzyme preparation (less than 10^4 cpm), SAM (0.05 μCi/0.5 μmole), phenolic substrate (0.5 μmole). The reaction mixture without SAM was preincubated for 5 min., then the SAM was added as a starter and incubated for 0.5–1.0 hr. at 30°C. The reaction was terminated by the addition of 5% of HCl (0.5 ml), and the products formed were extracted with ether. The extracts (first 10 ml, then 5 ml of ether) were combined and evaporated under reduced pressure. The residues were dissolved in dioxane (0.5 ml) and trans-

fered into a vial containing the scintillator (5 ml), then counted by a Beckmann LS100 scintillation counter. The counting effiency was 89%, and 10^4 cpm corresponded to 50.6 nmole of the methylated products formed.

Extraction of Bamboo OMT

Bamboo (*Phyllostachys pubescens*) young shoots were harvested in June, from which enzymes were instantly extracted in a cold room (4–6°C) as follows. The sliced shoots (6 kg) were homogenized with buffer A (4 *l*) by a domestic mixer, then the homogenate was squeezed with four-layered guaze. The juice obtained were centrifuged at 10^4 rpm for 20 min., and supernatant of which was precipitated by adding solid ammonium sulfate (finally 70%-saturation). The precipitate was stored at −20°C until for use.

Purification of bamboo OMT

Step 1: The stock preparation (33 ml) was centrifuged at 10^4 rpm for 15 min.

Step 2: The precipitate was dissolved in 55%-saturated ammonium sulfate-buffer C soln. (30 ml) with EDTA (150 μmole), and centrifuged at 10^4 rpm for 15 min. Then, the same procedure was repeated for the precipitate. The precipitate thus obtained was dissolved in 20%-saturated ammoium sulfate buffer C soln. (20 ml) which contained EDTA (100 μmole) and 5-hydroxyferulate (330 μmole) and centrifuged at 10^4 rpm for 15 min. The precipitate was again subjected to the same procedure. Subsequently, EDTA (250 μmole) and solid ammonium sulfate (17 g; finally 70%-saturation) were added to the supernatant and the precipitate was collected by the centrifugation. The precipitate was then passed through a Sephadex G25 column (40 cm × 2.8 cm; V_0=125 ml).

Step 3: DEAE-cellulose (6 g of dry weight, which was preliminary washed and bufferized with buffer C), was added to the enzyme soln. desalted (80 ml). The soln. was stirred for 20 min., and then the enzyme absorbed on the ion-exchange-cellulose was charged on a column (6 cmϕ). After washing it with buffer C (150 ml) and subsequently 0.05 M KCl-buffer C soln. (235 ml), the fraction eluted with 0.2 and 0.3 M KCl-buffer C were collected (182 ml).

Step 4: The combined fraction was precipitated by adding ammonium sulfate (finally 60%-saturation) and it was centrifuged at 10^4 rpm for 15 min. The precipitate was then dissolved in buffer C, and applied on to a Sephadex G200 column (flow rate 12 ml/hr., V_0=170 ml, solvent for the elution: buffer C). The fraction numbers from 32 to 54 were collected (71.5 ml) for further purification.

Step 5: The preparation was applied on a DEAE-cellulose column (bufferized with buffer C, 10 cm × 1.7 cmϕ). Then, the enzyme was successively fractionated by stepwise elution with following buffer solns: buffer C, 0.1 M-, 0.15 M-, and 0.25

M-buffer C soln. The fraction eluted by 0.15 M was collected, which was final preparation.

The pH was maintained by adding 1 N NH_4OH soln, during the ammonium sulfate precipitation. The second purification was almost the same as the procedure described above, except step 5 was operated using linear gradient elution.

Disc Electrophoresis

Disc electrophoresis was carried out by the method of Davis[15]. The gel after electrophoresis, was directly assayed for FA- and SA-activities.

Isoelectric Fractionation

According to the method of Vesterberg *et al.*[22], carrier Ampholyte was charged on an isoelectric forcusing-column (LKB-ampholine, 110 ml), where the final Ampholytes concentration was 1%. After charging sample (Step 5), a voltage (700 V; initial current ca. 8 mA) was applied for 36 hr. at 4°C.

Protein Determination

The sample for protein determination was dialyzed against buffer C. Subsequently, coloration with Lowry's phenol reagent was followed and protein was determined at 750 nm[14]. Protein contents were calculated by means of serum albumine-calibration curve. The net contents were obtained by subtracting the absorbance of the buffer C as a control.

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